

## Alamethicin-Mediated Fusion of Lecithin Vesicles

(nuclear magnetic resonance/model membranes/antibiotics/ion transport across membranes/voltage gateable ion channels)

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**ABSTRACT** It was recently shown that alamethicin greatly facilitates the fusion of small, sonicated, lecithin bilayer vesicles. In the present work the details of this fusion process have been followed by monitoring the inner and outer choline methyl signals separately by proton magnetic resonance spectroscopy. It is shown that during the alamethicin-induced fusion some of the antibiotic molecules become translocated from the extravesicular aqueous medium into the enclosed intravesicular space, and these alamethicin molecules were found to affect the choline methyl signals from the inner half of the bilayer only. No evidence was obtained for transmembrane coupling of the two halves of the bilayer in the presence of alamethicin or for any effects that might be construed as due to incorporation of alamethicin molecules into the hydrophobic core of the bilayer.

The interaction of alamethicin (1, 2) with phospholipid bilayers has been a subject under extensive investigation by various methods (3-6) ever since it was discovered that this antibiotic induces ion movement across biological membranes (7) and that it interacts with black lipid membranes to form voltage gateable ion channels with discrete conductance states (8).

Two nuclear magnetic resonance (NMR) studies of the interaction of alamethicin with phospholipid bilayers have appeared. Hauser *et al.* (9) investigated the interaction of alamethicin with sonicated phospholipid bilayer vesicles and found that the oligopeptide causes broadening of the lipid proton resonances as well as reduction in the signal intensities. These workers interpreted the data in terms of incorporation of alamethicin into the hydrophobic core of the bilayer membrane. A more recent study (10), however, indicated that alamethicin interacts with lecithin bilayer vesicles primarily in the region of the polar head groups. The observed intensity loss and linewidth broadening effects were attributed to fusion of bilayer vesicles catalyzed by clusters of alamethicin in addition to specific interactions between the antibiotic molecules and the polar head groups. The following model was also proposed for this alamethicin-induced vesicle fusion process. It was suggested that clusters of alamethicin, which become physically adsorbed onto the surface of the bilayer vesicles, readily transcend the thickness of the electrical double layer of these colloidal particles and, hence, could serve as a nucleation center for the fusion of two bilayer units. Presumably these alamethicin clusters can, during the course of fusion, cause transient rearrangements in the local structure of the

lipids, with the resultant formation of an inverted micelle and the subsequent translocation of the alamethicin cluster into the intravesicular solution. A schematic representation of the sequence of events proposed for this fusion process is reproduced in Fig. 1.

The present work was undertaken to test the validity of the proposed model of alamethicin-mediated fusion. Our rationale is that the translocation of the alamethicin into the intravesicular solution could be ascertained by studying the effect of externally added alamethicin on the choline head groups located on the inner and outer halves of the bilayer separately. In the proton magnetic resonance (PMR) spectrum of lecithin bilayer vesicles, the choline methyl protons give rise to two partially resolved signals at about  $-3.2$  ppm due to the different surface curvatures of the outside and inside surface of the bilayer unit. Although the small chemical shift difference between these two overlapping signals (about 5 Hz at 51.7 kG, depending on temperature) does complicate the analysis of the spectral data, complete resolution of these choline signals is possible when the experiments are carried out in the presence of certain paramagnetic shift reagents in the extravesicular medium, as demonstrated by Bystrov and coworkers (11). More recently, Levine *et al.* (12), in fact, showed that it is possible to completely resolve the two choline signals without significant alteration of the linewidths using appropriate quantities of europium(III) ions. This latter technique will be exploited in the present work.

### EXPERIMENTAL

**Materials.** Alamethicin was obtained from Dr. G. B. Whitfield, Jr., of the Upjohn Co. (Sample nos. U-22324, 8831-CEM-93.1 and 93.3) and was used without further purification.

L- $\alpha$ -Dipalmitoyl lecithin was purchased from General Biochemicals and purified according to a published procedure (13). D<sub>2</sub>O (99.8%) was purchased from Stohler Isotope Chemicals. CH<sub>3</sub>OH and CHCl<sub>3</sub> (used in column chromatography for the purification of lecithin) were MCB spectrograde products. NaCl was Baker analyzed reagent product. Europium nitrate hexahydrate [Eu(NO<sub>3</sub>)<sub>3</sub>·6H<sub>2</sub>O] was obtained from K & K Laboratories. All materials were used as received.

**Sample Preparation.** Sonicated bilayer vesicles (average size 250-300 Å) were prepared with a Branson sonifier equipped with a microtip. A known quantity of purified lecithin was weighed into a centrifuge tube, to which D<sub>2</sub>O containing 2 mM phosphate (sodium salt) and 0.1 M NaCl at pD 7.4 was added to give a suspension of about 50 mg of lecithin per ml of D<sub>2</sub>O.

Abbreviations: NMR, nuclear magnetic resonance; PMR, proton magnetic resonance.

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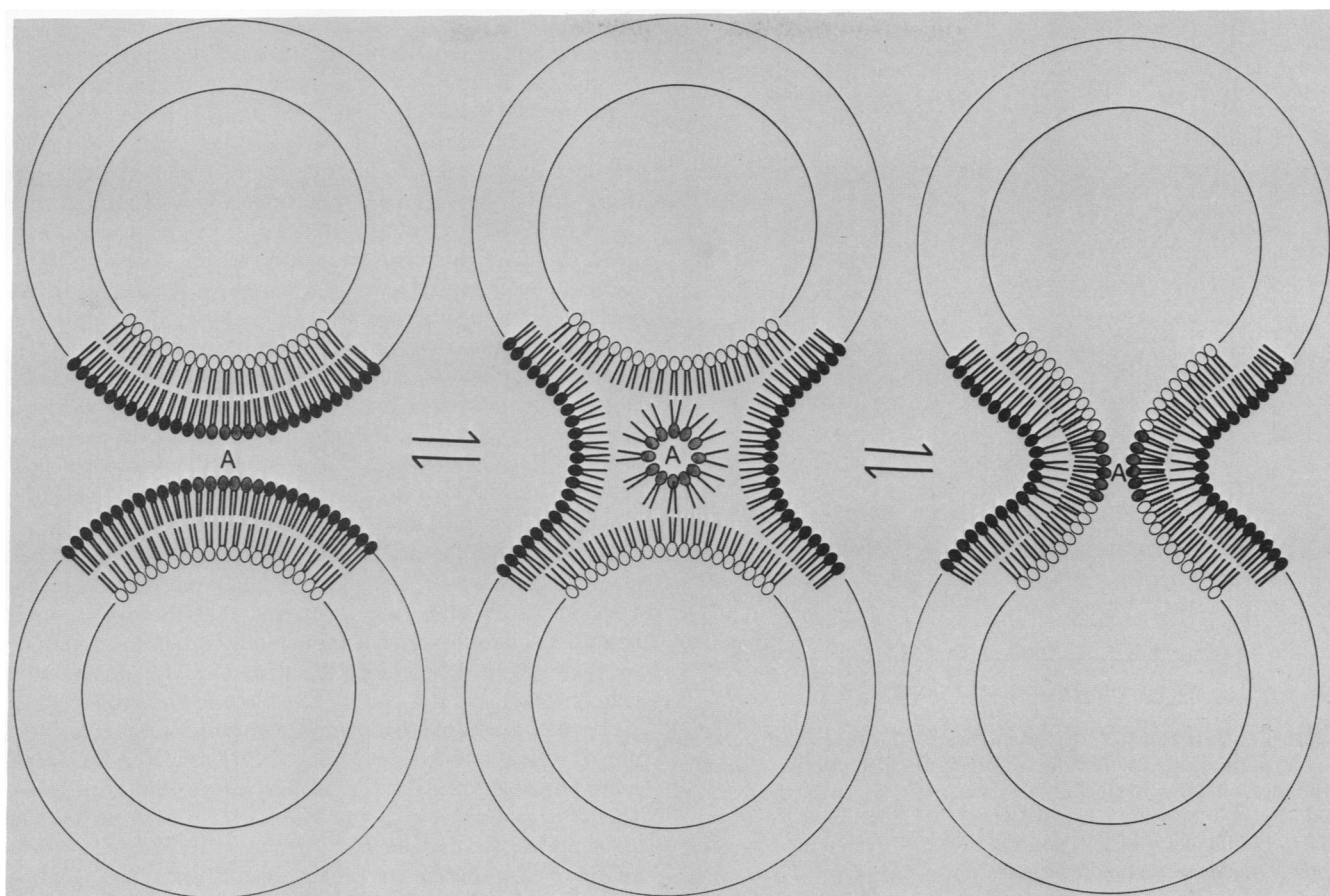


Fig. 1. Schematic representation of a possible sequence for the alamethicin-mediated fusion of lecithin vesicles. A = alamethicin aggregate (not to scale).

Sonication with high power for about 15 min gives a nearly transparent, colorless solution. This was centrifuged at 12,000 rpm in a superspeed RC-2 centrifuge for 40 min to remove residual multilayers from the small vesicles suspended in the supernatant. The latter was used in all subsequent NMR and electron microscope experiments.

Alamethicin was weighed into a 1-ml volumetric flask and dissolved in an appropriate amount of D<sub>2</sub>O containing 2 mM phosphate and 0.1 M NaCl at pD 7.4. Europium nitrate and sodium chloride were weighed into a 25-ml volumetric flask, to which D<sub>2</sub>O containing 2 mM phosphate at pD 7.4 was added so that the resulting solution was isotonic with the vesicle solution.

In one series of experiments, known quantities of alamethicin and vesicle solutions were mixed and incubated at 70° for 20 min. To the incubated mixture a measured amount of the europium solution was then added, followed immediately by NMR and electron microscope experiments.

In another series of experiments, the europium solution was mixed with the sonicated vesicle solution *before* the addition of alamethicin. After alamethicin was added, the time course of the PMR spectrum of the inner and outer choline groups was followed by Fourier transform-NMR spectroscopy.

Finally, the effect of alamethicin on a lecithin vesicle suspension sonicated in the presence of europium(III) ion was also studied. To the sonicated mixture a small quantity of alamethicin (about 0.01%) was added. After fusion had subsided, the mixture was dialyzed at room temperature against

an isotonic NaCl solution for 4 hr. Subsequent NMR and electron microscope experiments were performed on the dialyzed mixture.

**PMR Spectra.** The Fourier transform spectra of sonicated lecithin bilayer vesicles were obtained with a Varian XL-100 NMR spectrometer equipped with Fourier transform accessories and interfaced to a Varian 620i computer. Intensities of the signals were calibrated against a standard chloroform capillary treated with the free radical 2,2-diphenyl-1-picrylhydrazyl. Chemical shifts were measured against an external tetramethylsilane (TMS) capillary without correction for changes in the bulk magnetic susceptibility. Sample temperature was maintained by a Varian 4540 temperature control unit, and was determined from the spectrum of a standard ethylene glycol sample.

**Electron Microscopy.** Electron microscopy has been used to ascertain the size distribution of vesicles in the absence and presence of the paramagnetic ion and/or alamethicin. Specimens were prepared according to a procedure described in an earlier paper (10), and were observed on a Philips 201 electron microscope operating at 60 kV.

## RESULTS AND DISCUSSION

**Effect of Alamethicin-Induced Fusion on the Inner and Outer Choline Head Groups.** The effect of alamethicin-induced fusion on the inner and outer choline groups can be ascertained if appropriate amounts of europium(III) ions are added to the

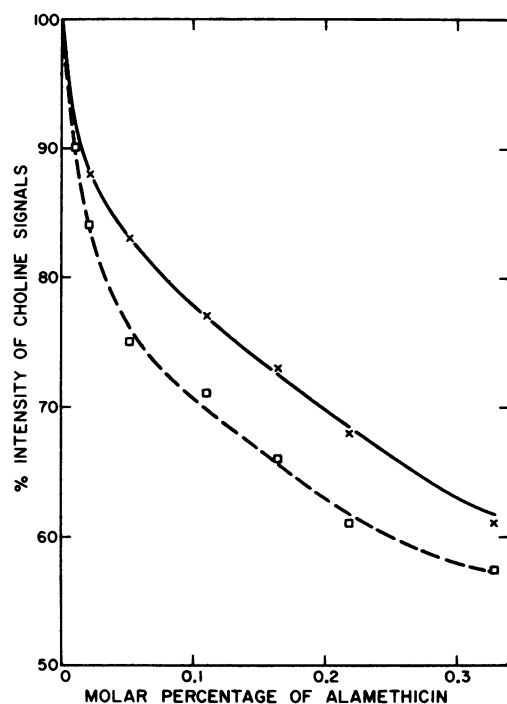


FIG. 2. Variations of the intensities of the inner and outer choline methyl signals of sonicated dipalmitoyllecithin vesicles as a function of alamethicin concentration. *Solid line* is for the outer choline signal; *dashed line* is for the choline signal from the inner half of the bilayer vesicle. Data have been corrected for changes in the stoichiometric lipid concentration as a result of the vesicle fusion. Alamethicin concentration is expressed as molar percentage of the phospholipid in the dispersion.

solution after fusion has taken place. The added europium(III) ions shift the outer choline methyl signal to higher fields in the PMR spectrum, thus permitting the inner and outer choline signals to be monitored separately.

Table 1 summarizes the effect of alamethicin and its associated induced-fusion on the intensities and linewidths of the inner and outer choline signals. The observed variation of the signal intensities with alamethicin concentrations is also presented in Fig. 2. Since variations in the size distribution of the vesicles during the fusion process change the stoichiometric

TABLE 1. Variations in intensity and linewidth of the inner and outer choline signals in the presence of alamethicin\*

Alamethicin (molar %)	Outer choline		Inner choline	
	Line- width (Hz)	Intensity (%)	Line- width (Hz)	Intensity (%)
No alamethicin	5.6	100	4.6	100
0.02	6.4	88	5.4	84
0.06	7.0	83	6.0	75
0.11	8.6	77	7.8	71
0.16	~9	73	~10	66
0.22	~10	68	~10.5	61

\* Europium(III) ions (7 mM) added to extravesicular solution only. The vesicles were fused for 20 min at 70°. Intensities are expressed in terms of lipid concentrations expected for each half of the bilayer for the size distribution of vesicles determined at the time the NMR experiments were performed.

concentrations of lipids in the two halves of the bilayer, proper interpretation of these intensity data can only be made after the transfer of lipid from the outer to the inner half of the bilayer is taken into account. Accordingly, these intensities are expressed in terms of the percentage of lipid expected for each half of the bilayer for the size distribution of bilayer vesicles determined (by electron microscopy) at the time the NMR measurements were performed. Both the inner and the outer choline signals exhibit a decrease in intensity as the alamethicin concentration in the sample increases, in accordance with results reported earlier (9, 10). We note, however, that despite their supposedly shielded location from the external solution, the choline methyl protons from the inner half of the bilayer actually manifest a somewhat greater reduction in their PMR signal intensity. This result, we believe, provides evidence that some of the antibiotic molecules become incorporated into the intravesicular space during the alamethicin-mediated fusion process.

Intensity loss in lipid bilayer vesicle systems arises from magic angle effects. The local lipid chain motion in a bilayer vesicle is often sufficiently restricted that the dipolar interactions among spins are not completely averaged out spatially by these segmental motions. That part of the dipolar interactions not averaged out by the local motion can only be motionally averaged by overall rotational tumbling of the entire vesicle unit and/or lateral diffusion of the lipid molecules on the surfaces of the bilayer vesicle. The timescale of this motional narrowing can be shown to have a profound influence on the lineshape of the spectrum. For example, in multilamellar dispersions where overall rotational tumbling of the bilayer unit and the timescale for surface diffusion of the lipid molecules (measured in terms of root mean square angle traversed per unit time) is slow, the lineshape of the multilayer spectrum is dominated by inhomogeneous broadening due to these residual dipolar interactions (14, 15). However, when the modulations of these residual fields are sufficiently fast, one can obtain a homogeneously broadened resonance which accounts for all of the expected intensity. This presumably is the situation with small sonicated lecithin bilayer vesicles (250–300 Å in diameter), where the local lipid chain motion appears to be sufficiently unrestricted and the overall tumbling of the bilayer unit is fast enough (16). Accordingly, no intensity anomaly is observed for these vesicles above the phase transition temperature. Large bilayer vesicles are intermediate between these two limits. Here the modulation of the residual dipolar interactions can only be effectively averaged by surface diffusion of the lipid molecules, but the rate of this motional narrowing is in general not sufficiently rapid compared with the range of residual interactions (expressed in frequency units) that must be averaged. This leads to magic angle effects as lipid molecules with chain axes located near the magic angle ( $54^{\circ}44'$ ) are more effectively averaged compared to those further away. The lineshape of the NMR signal is, therefore, not completely homogeneous, and the high-resolution PMR spectrum can exhibit an apparent intensity anomaly because only those protons belonging to chains that happen to be oriented within a certain cone about the magic angle which the chain axes make with respect to the applied magnetic field are sufficiently narrow to appear in the high-resolution spectrum.

There are two possible reasons for the intensity reduction observed for the choline signals when alamethicin is added to

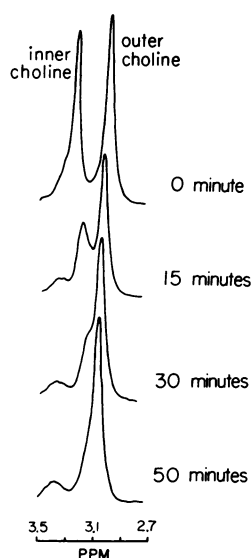


FIG. 3. Time evolution of the 100 MHz PMR spectra of the choline methyl protons when the alamethicin-mediated fusion is carried out in the presence of 7 mM europium(III) ion in the extravesicular solution. The antibiotic is added to the medium at 0 min. Incubation temperature: 70°. Alamethicin concentration: 0.2%. Chemical shift measured downfield from an external tetramethylsilane (TMS) signal without making bulk susceptibility corrections. The broad resonance at 3.4 ppm is due to the  $\text{Me}_3\text{N}-\text{CH}_2$  protons.

these lecithin vesicle solutions. First, the alamethicin-induced fusion leads to an increase in the vesicle size distribution, which results in slower overall rotational tumbling of the bilayer units as well as less effective averaging by surface diffusion of the lipid molecules. Second, alamethicin can also interact with the polar head group region of the lecithin bilayer, with a resultant decrease in flexibility and mobility of the choline head groups. In the present experiment, both factors contribute to the apparent intensity anomaly since it has previously been shown that the motion of these choline head groups is essentially unrestricted and rapid even for large vesicles approximately 1000 Å in diameter at temperatures over 40°C (16). Magic angle effects are not observed for the choline proton resonance of these large vesicles.

**Translocation of Paramagnetic Ions Across the Bilayer Membrane During Alamethicin-Mediated Fusion.** In the previous section evidence was presented for the entrapment of alamethicin molecules into the intravesicular solution as a consequence of the alamethicin-mediated fusion. Since alamethicin is negatively charged, we expect, as a corollary, that counterions will be translocated as well. This point could be ascertained if, for example, europium(III) ions were present in the extravesicular bilayer vesicle solution prior to the onset of the alamethicin-induced fusion. A series of experiments was therefore carried out under these conditions.

The results of this study are summarized in Fig. 3, where the choline spectrum is displayed as a function of time after the addition of alamethicin to the solution. These data show a continuous upfield shift of the inner choline signal relative to the outer choline resonance accompanied by a concomitant intensity reduction as the fusion process progressed, until it finally merged completely with the outer choline signal some 50 min after the addition of alamethicin. Since the intra-

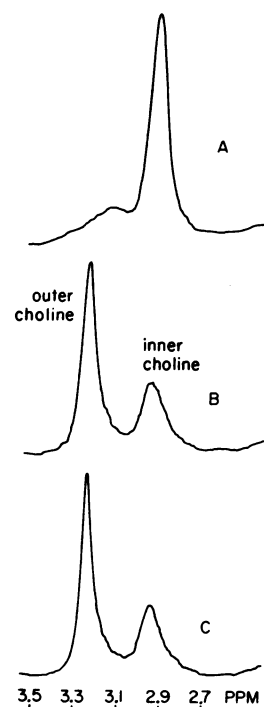


FIG. 4. 100 MHz PMR spectra of the choline methyl protons of lecithin vesicles after fusion by 0.01% alamethicin in the presence of 11 mM  $\text{Eu}^{3+}$  in both the intra- and extravesicular media (A); and after entrapment of alamethicin and europium ions in the intravesicular solution (B and C). Spectrum (B) was obtained after dialysis of the solution for (A) against NaCl solution for 4 hr. Spectrum (C) was obtained after incubation of the solution for (B) at 70° for an additional 2 hr.

vesicular solution contained no europium(III) ions to begin with, the observed upfield shift of the inner choline signal must arise from the translocation of europium(III) ions during the fusion process, or be the result of alamethicin pores formed across the bilayer membrane. In a separate experiment we have shown that lecithin bilayers are impermeable to europium(III) in the absence of alamethicin even after incubation of the vesicles at 70°C for over 3 hr. In the next section, we shall also rule out the possibility of alamethicin pores, and hence the rapid translocation of europium(III) must be associated with the fusion process.

**Entrapment of Alamethicin in Small Sonicated Vesicles.** Alamethicin could be trapped in small vesicles when the antibiotic-induced fusion is carried out at low antibiotic concentrations (about 0.01%) followed by removal of the external alamethicin and ions by dialysis. Under these conditions only one or two fusion steps on the average could have occurred for each of the initial vesicles, so that the sizes of the resultant fused vesicles are still rather small. The concentration of alamethicin trapped within the intravesicular space is quite high since the surface-to-volume ratio of the enclosed solution remains high. When the vesicles are prepared by sonication in the presence of europium(III) ions, followed by subsequent fusion and dialysis of the external medium, a europium(III) ion gradient as well as an alamethicin gradient is also established across the bilayer membrane. These asymmetrical bilayer vesicles could, therefore, be used to ascertain whether there is transmembrane coupling between the two halves of the bilayer and whether alamethicin pores are present within

the bilayer membrane to an appreciable extent to permit significant leakage of entrapped europium(III) ions.

The results of such an experiment are summarized in Fig. 4. Because of the trapped europium(III) ions, the PMR spectrum of these vesicles gives two separate choline resonances, with the inner choline proton signal appearing at higher field. The inner choline signal was found to be a factor of two or so broader than the outer choline signal. Its integrated intensity was also significantly less than expected. Electron microscopy revealed that the average diameter of these vesicles was about 300 Å, and thus the ratio of the outer compared with the inner lipid concentrations should be about 1.6, but the actually observed intensity ratio was 1.9. This intensity result, together with the observed broadening of the inner choline resonance, indicate that there is reduction in the motional flexibility and mobility of these polar head groups as a result of the alamethicin-lipid interactions. Since no significant broadening (or intensity loss) of the outer choline signal was observed, interactions of alamethicin with the bilayer at the inner lipid-aqueous interface are not transmitted to the outer half of the bilayer.

The above spectral results provide confirming evidence in support of our earlier hypothesis that alamethicin molecules are translocated into the intravesicular solution during the mediated fusion process. Any interaction of alamethicin with lecithin bilayers appears to be confined principally at the bilayer-aqueous interface. Presumably any appreciable incorporation of the polypeptide within the hydrophobic core of the bilayer would have led to transmembrane coupling of the two halves of the bilayer. No evidence was obtained for this transmembrane coupling. Our experiments showed that the PMR spectrum of these choline head groups in alamethicin-containing vesicles was essentially unchanged both in chemical shifts and relative intensities even after an incubation period of about 2 hr at 70°C. This result indicates that there is no appreciable leakage of the entrapped alamethicin or europium(III) ions into the extravesicular solution, sug-

gesting that there is no extensive alamethicin pore formation under the conditions of these experiments.

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